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Use of a Bacteriocin Produced by *Pediococcus acidilactici* To Inhibit *Listeria monocytogenes* Associated with Fresh Meat

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A bacteriocin produced by *Pediococcus acidilactici* had an inhibitory and bactericidal effect on *Listeria monocytogenes* associated with fresh meat. MICs were significantly lower than minimum killing concentrations. When meat was inoculated with *L. monocytogenes*, the bacteriocin reduced the number of attached bacteria in 2 min by 0.5 to 2.2 log cycles depending upon bacteriocin concentration. Meat treated initially with the bacteriocin resulted in attachment of 1.0 to 2.5 log cycles fewer bacteria than that attained with the control. The bacteriocin, after 28 days of refrigerated storage on meat surfaces, was stable and exhibited an inhibitory effect on *L. monocytogenes*.

Recent outbreaks of food-borne listeriosis have caused much concern in the food industry. *Listeria monocytogenes* infects a small proportion of the population, such as pregnant women, newborn children, and persons with an immunocompromised condition (9, 14, 16, 23). However, among those infected, the fatality rate is high, ranging from 27 to 34% (9, 14, 16). Because of the high fatality rate, listeriosis is estimated to cost much more per case than more common but less serious food-borne illnesses (20).

Although reported outbreaks of listeriosis have been traced to milk, cheese, cabbage, and poultry (2, 9, 14, 16), these are not the only sources of *L. monocytogenes* in the environment. It has been isolated from soil, water, feed, and animal feces (18, 22). Additionally, *L. monocytogenes* has been isolated from fresh beef and poultry, processed meats, and numerous vegetables in retail outlets (8, 22). Because of its widespread occurrence in nature, the potential for post-processing contamination of foods with *L. monocytogenes* is high.

Many lactic acid-producing bacteria are used to preserve a variety of foods by lowering the pH. Some of these bacteria produce inhibitory compounds in addition to lactic acid. One such organism, *Pediococcus acidilactici*, produces a bacteriocin which, in a crude, dried form, has been shown to inhibit *L. monocytogenes* in some dairy products (15). The antimicrobial activity of this bacteriocin on a beef carcass has not been demonstrated. The purpose of this project was to determine if the bacteriocin would reduce populations of *L. monocytogenes* already attached to a carcass and remain stable enough to inhibit further contamination which could occur during processing.

MATERIALS AND METHODS

Bacterial strains. *Listeria monocytogenes* Scott A and *Listeria ivanovii* KC 1714 were obtained from the Division of Microbiology, U.S. Food and Drug Administration, Cincinnati, Ohio. *P. acidilactici* was from a commercial culture, LACTACEL 110, provided by Microlife Technics, Sarasota, Fla. Cultures of *Listeria* spp. were maintained on tryptic soy agar (TSA) slants (Difco Laboratories, Detroit, Mich.). Before use, cultures were grown for 24 h in tryptic soy broth (TSB) at 23°C. *P. acidilactici* was maintained in MRS broth (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 2% yeast extract (Difco) (15). *L. monocytogenes* was enumerated by plate counts on TSA by the pour plate method with 48 h of incubation at room temperature. Dilutions were prepared in accordance with accepted procedures (1).

Meat. Fresh lean beef muscle was obtained from the abattoir at the U.S. Meat Animal Research Center. Muscles were cut into 0.5-cm-thick slices, sterilized with gamma radiation (42 kGy of ^{60}Co at −40°C), vacuum packaged, and stored at −15°C. Before use, slices of meat were thawed at room temperature and cut with a sterile scalpel into pieces (1.0 by 1.0 cm), giving the samples a surface area of 4 cm^2 each.

Preparation of bacteriocin. *P. acidilactici* was grown for 18 h at 37°C in 100 ml of MRS broth supplemented with 2% yeast extract. Cells were removed by centrifugation at 5,000 × *g* for 15 min at 4°C in a J-6B centrifuge (Beckman Instruments, Inc., Palo Alto, Calif.). The supernatant fluid was collected, neutralized to pH 7.0 with NaOH (to prevent any acid inhibition), filter sterilized by being passed through a sterile 0.2-μm-pore-size filter (Gelman Sciences, Inc., Ann Arbor, Mich.), and stored at 5°C.

Determination of bacteriocin concentration. Serial twofold dilutions were made with the filter-sterilized bacteriocin solution and sterile water. From each dilution, 5 μl was placed on the surface of a semisolid TSA overlay (TSB with 0.75% agar) seeded with *L. ivanovii*, the sensitivity indicator organism. Plates were incubated overnight at room temperature. One arbitrary unit (AU) was defined as 5 μl of the highest dilution of the bacteriocin solution causing a definite zone of inhibition on the lawn of the indicator organism.

Determination of MICs and MKCs. *L. monocytogenes* and *L. ivanovii* were grown overnight at room temperature in TSB. The sterile bacteriocin solution was diluted to an initial concentration of 1,024 AU/ml. Serial twofold dilutions were then made in 1-ml volumes of TSB down to 1.0 AU/ml. To each tube, approximately 10^3 bacteria were added, and the tubes were incubated 24 h at room temperature. The MIC was determined as the lowest concentration of bacteriocin which prevented visible turbidity in tubes after 24 h. Each tube showing no turbidity after 24 h was vortexed, and 0.1 ml of its contents was transferred to 10 ml of TSB and incubated for 24 h at room temperature. Minimum killing concentration

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(MKC) was defined as the lowest original concentration allowing no visible turbidity in tubes after 24 h.

Inhibition of *L. monocytogenes* attached to meat. Cut meat pieces were soaked in 20 ml of Butterfield phosphate buffer (21) containing a large inoculum (10^7 CFU/ml) or a small inoculum (10^4 CFU/ml) of *L. monocytogenes* for 10 min. Samples were then removed and placed into a sterile beaker containing 20 ml of bacteriocin solution for 0, 2, 5, or 10 min. All samples were gently rinsed in sterile saline solution (0.87% NaCl). Cells remaining on the meat after it was rinsed were considered to be attached. Samples were then placed into a sterile bag containing phosphate buffer and blended for 2 min in a Stomacher 400 (Techmar Inc., Cincinnati, Ohio). Counts were determined by the pour plate method on TSA.

Inhibition of *L. monocytogenes* during attachment. Cut meat pieces were soaked in 20 ml of sterile bacteriocin solution for 10 min. The concentrations of bacteriocin were 500, 1,000, and 5,000 AU/ml. The samples were then placed into a beaker containing 20 ml of bacteria suspended in phosphate buffer for 0, 2, 5, or 10 min. The bacterial populations of the inocula were 10^7 or 10^4 CFU/ml. The samples were then rinsed gently in sterile saline solution, placed into sterile bags containing phosphate buffer, and blended for 2 min in the stomacher. Populations were determined by the pour plate method on TSA.

Effect of storage on bacteriocin activity. Pieces of meat (1.0 by 1.0 by 0.5 cm) were placed into the three bacteriocin solutions described above for 10 min. The samples were transferred to a sterile beaker which was then covered with Parafilm (American Can Company, Greenwich, Conn.) and stored at 5°C. At intervals of 0, 7, 14, 21, and 28 days, one sample from each container was placed into 10 ml of a bacterial solution containing approximately 10^5 CFU/ml for 5 min. Samples were then gently rinsed in sterile saline solution, placed into sterile bags containing phosphate buffer, and blended for 2 min in the stomacher. Populations were determined by the pour plate method on TSA.

Statistical analyses. In all tests, five independent replications were performed in duplicate. Data from MICs and MKCs were analyzed by paired *t* tests. All inhibitory tests involving meat were set up as split plots with times as whole unit treatments in a randomized complete block design. Bacteriocin concentrations were applied to subunits. An analysis of variance on a subunit basis was conducted to determine differences (4).

RESULTS

Bacteriocin concentration. The assays revealed a bacteriocin concentration of 51,200 AU/ml with *L. ivanovii* as the sensitivity indicator organism. The dilution method employed yielded the same bacteriocin concentration for each assay.

MICs and MKCs. Values were determined for both *L. monocytogenes* and *L. ivanovii*. The averages were 460 AU/ml (MIC) and 1,024 AU/ml (MKC) for *L. monocytogenes* and 25.6 AU/ml (MIC) and 192 AU/ml (MKC) for *L. ivanovii*.

Inhibition of *L. monocytogenes* attached to meat. The effect of the bacteriocin on *L. monocytogenes* attached to meat is shown in Fig. 1. When a large inoculum (Fig. 1a) was used, the smallest concentration of bacteriocin (500 AU/ml) reduced the number of *L. monocytogenes* by less than 1 log cycle. When a bacteriocin concentration of 1,000 AU/ml was used, the population decreased by slightly more than 1 log cycle in 10 min. At a concentration of 5,000 AU/ml, the bacteriocin reduced the population by 2 log cycles, with most of the reduction occurring in the first 2 min.

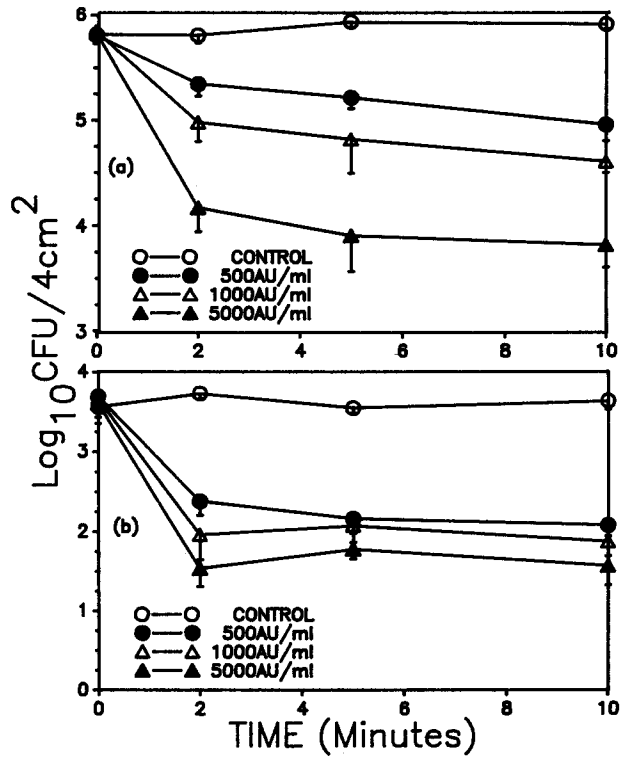

FIG. 1. Effect of the bacteriocin on *L. monocytogenes* when the bacteriocin was applied after meat had been contaminated with a large inoculum (a) or a small inoculum (b). Values are the averages of five independent trials. Vertical bars represent the standard error of the mean.

When a smaller inoculum (Fig. 1b) was used, all concentrations of bacteriocin reduced the number of bacteria attached to meat by 1 to 2 log cycles within 2 min. The change in numbers after 10 min was not significantly different ($P > 0.05$) from that which occurred after 2 min.

Inhibition of *L. monocytogenes* during attachment. Sterile pieces of meat were treated with different concentrations of the bacteriocin before exposure to *L. monocytogenes*. The toxic effect of the bacteriocin resulted in fewer bacteria being able to attach to the treated meat than to the untreated control. With a large inoculum (Fig. 2a), the decrease in the number of attached bacteria ranged from 1 to 2 log cycles after 2 min of exposure to the bacteria.

When treated meat was exposed to a small inoculum, few bacteria became attached to the meat. Fewer than 100 CFU/4 cm^2 were attached to the meat after 2 min, compared with more than 3,500 CFU/4 cm^2 for the control (Fig. 2b).

Effect of storage on bacteriocin activity. Compared with the control, the bacteriocin at a concentration of 5,000 AU/ml reduced the number of bacteria attaching to the meat by as much as 2.7 log cycles after 7 days and 1.2 log cycles after 28 days (Fig. 3). At a concentration of 1,000 AU/ml, the inhibition exhibited by the bacteriocin was slightly less, dropping to below 1 log cycle after 28 days. A bacteriocin concentration of 500 AU/ml resulted in attachment of less than 1 log cycle fewer bacteria than were attached to the control after only 7 days of storage. The difference was not significant ($P < 0.05$).

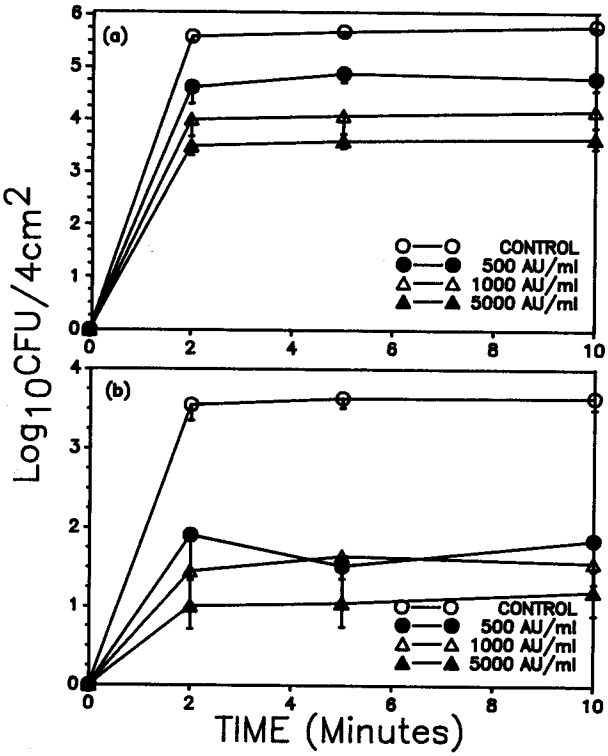

FIG. 2. Effect of the bacteriocin on *L. monocytogenes* when bacteriocin was applied to meat first and was exposed to a large inoculum (a) or a small inoculum (b). Values are the averages of five independent trials. Vertical bars represent the standard error of the mean.

DISCUSSION

L. monocytogenes has been shown to be able to grow under a variety of conditions. It is able to survive and grow on meat and meat products at refrigeration temperatures (3, 11, 17). The organism is able to survive in acid conditions with pHs as low as 4.8 in foods (5, 11) and as low as 4.4 in laboratory media (19). In addition, *L. monocytogenes* is able to survive high-temperature, short-time pasteurization (71.7°C, 15 s), possibly protected inside polymorphonuclear leukocytes (7). Pasteurized milk was implicated in an outbreak of listeriosis in 1983, but this was not confirmed absolutely (9). Traditional methods of preservation such as refrigeration, pasteurization, and low pH are not completely effective in controlling or eliminating *L. monocytogenes* from food.

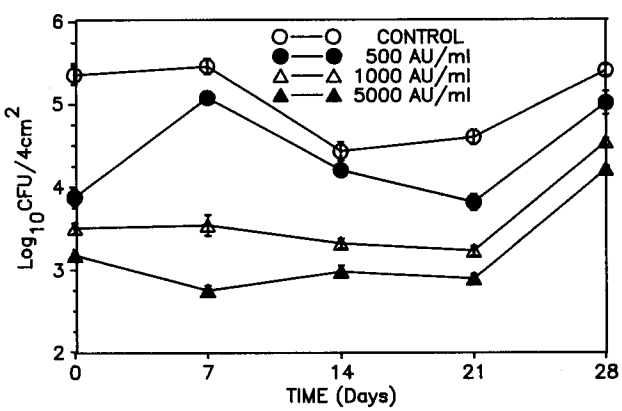

FIG. 3. Inhibition of *L. monocytogenes* after storage of bacteriocin-treated meat at 5°C. Values are the averages of five independent trials. Vertical bars represent the standard error of the mean.

Some of the lactic acid bacteria produce, in addition to lactic acid, bacteriocins which further inhibit *L. monocytogenes*. The bacteriocins associated with different *Pediococcus* species are encoded on plasmids. Plasmids associated with bacteriocin production in different pediococci are of different sizes (6, 12, 13). Pucci et al. (15) investigated the effect of the bacteriocin from *P. acidilactici* PAC 1.0 on *L. monocytogenes* in different foods. They found the bacteriocin to be inhibitory toward *L. monocytogenes* at different temperatures and pH values.

The results of this study reveal that the bacteriocin produced by *P. acidilactici* is inhibitory toward *L. monocytogenes* in association with red meat. The effectiveness of the bacteriocin appears to be dependent upon concentrations of both bacteriocin and bacteria.

The log cycle reduction in number of bacteria attached to meat was greater when a smaller inoculum was used to contaminate the meat before treatment with the bacteriocin. The smaller inoculum (10^3 to 10^4 CFU/ml) is probably closer to the size of the inoculum which would be encountered if contamination occurred during processing. The lowest concentration of bacteriocin (500 AU/ml) reduced the number of attached bacteria by more than 1 log cycle when a smaller inoculum was used.

When meat was treated with the bacteriocin before exposure to *L. monocytogenes*, the number of bacteria which became attached to the meat was less than that observed with the untreated control. Again, the inhibition was greater when a small inoculum was used.

The data presented also indicate that treatment of meat with the bacteriocin before exposure to *L. monocytogenes* was more effective than application after contamination. A comparison of Fig. 1b and 2b reveals that the controls had the same numbers but that meat treated initially with the bacteriocin had fewer bacteria attached than did meat which was contaminated first. This also occurred when the large inoculum was used (Fig. 1a and 2a). This may have occurred because the injured cells were not able to attach to the meat, while cells already attached were able to remain attached and recover to form colonies in the nonselective plating medium. The differences between MICs and MKCs support this hypothesis. The meat may also have afforded a degree of protection to attached bacteria and thus reduced the effect of the bacteriocin.

Meat treated with the bacteriocin and stored at 5°C was not as heavily contaminated with *L. monocytogenes* as was the untreated control. There was a gradual decline over time in the amount of inhibition exhibited. The meat was stored in a beaker with a Parafilm cover, so little, if any, moisture evaporated. Fluid from the meat collected at the bottom of the beaker, and since the bacteriocin was dissolved in the fluid, there may have been a diluting effect due to the loss of this fluid. The same phenomenon would occur in a processing situation, so it cannot be ignored. The amount of inhibition afforded by the lowest bacteriocin concentration (500 AU/ml) dropped to less than half a log unit after 7 days, so it would be considered unsatisfactory.

There appeared to be no inactivation of the bacteriocin by the meat. The pH of meat, 5.3 to 5.7 (10), is within the range at which *L. monocytogenes* is able to grow and also within the effective pH range of the bacteriocin. The effect of the

bacteriocin was immediate. Most of the inhibition occurred within 2 min, with little additional inhibition occurring after that. This suggests that the bacteriocin was irreversibly bound to the bacterial cell, a hypothesis which will be investigated further.

The bacteriocin produced by *P. acidilactici* has been shown to inhibit or eliminate *L. monocytogenes* on meat surfaces when used in reasonably low concentrations. An accurate comparison of effective concentrations between this study and that of Pucci et al. (15) is not possible because of the arbitrary measurements, different sensitivity indicators, and different methods of diluting the bacteriocin. Additional studies are needed to purify the bacteriocin and characterize it so that accurate measurements and comparisons can be made and standard procedures can be developed.

Bacteriocins from lactic acid-producing bacterial starter cultures are present in some naturally fermented food products, so their addition to foods in a purified form should pose no risk to consumers. Although bacteriocins are probably safe, they are not yet approved as food additives. The use of bacteriocins in combination with traditional methods of preservation in close conjunction with good manufacturing procedures and strict sanitation could be effective in controlling *L. monocytogenes* on fresh meat, thus assuring a safer product for consumption or further processing.

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